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Isomeric fluorophenols were used as phenol analogues to investigate the transformation of phenol to benzoate by an anaerobic, phenol-degrading consortium derived from freshwater sediment. Transformation of 2-fluorophenol and 3-fluorophenol occurred in the presence or absence of phenol and led to the accumulation of fluorobenzoic acids. Identification of the resulting fluorobenzoate products as 3-fluorobenzoate and 2-fluorobenzoate isomers, respectively, together with the nontransformation of 4-fluorophenol indicated that the carboxyl group was introduced para to the phenolic hydroxyl group.

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ANAEROBIC TRANSFORMATION OF PHENOL TO BENZOATE VIA *para*-CARBOXYLATION: USE OF FLUORINATED ANALOGUES TO ELUCIDATE THE MECHANISM OF TRANSFORMATION[+]

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SUMMARY: Isomeric fluorophenols were used as phenol analogues to investigate the transformation of phenol to benzoate by an anaerobic, phenol-degrading consortium derived from freshwater sediment. Transformation of 2-fluorophenol and 3-fluorophenol led to the accumulation of fluorobenzoic acids. 2-Fluorophenol was transformed in the presence or absence of phenol, while 3-fluorophenol transformation was only observed in the presence of phenol. Identification of the resulting fluorobenzoate products as 3-fluorobenzoate and 2-fluorobenzoate isomers, respectively, together with the nontransformation of 4-fluorophenol indicated that the carboxyl group was introduced *para* to the phenolic hydroxyl group. © 1989 Academic Press, Inc.

Formation of benzoate from phenol and CO_2 in sewage sludge when H_2 was present was recently reported (1). While investigating anaerobic degradation of monochlorophenols, we have observed the conversion of phenol to benzoate after reductive dechlorination and before mineralization to CO_2 and CH_4 (2,3). To examine the nature of this reaction in more detail, we elected to use fluorine-substituted phenols as analogues of phenol recognizing that fluorinated substrate analogues can be of value in investigating metabolic pathways. As a result of this study, we have identified fluorine-substituted reaction products which show that carboxylation occurs at the *para* position. By analogy, we presume that phenol is carboxylated similarly.

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Abbreviations used: HPLC, high performance liquid chromatography; UV, ultraviolet light; GC-MS, gas chromatographic mass spectral analysis.

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MATERIALS AND METHODS

Bacterial Culture: A 2-chlorophenol-degrading anaerobic consortium (3) derived from freshwater sediment was diluted (10^{-1} to 10^{-9}) into phenol (4mM) medium. The 10^{-9} dilution culture which transformed phenol to benzoate with subsequent degradation of benzoate to CO_2 and CH_4 was used as inoculum. The consortium was maintained by replenishing the phenol every two weeks and transferring (10%) to fresh medium every six weeks.

Anaerobic Cultivation Methods and Medium: The anaerobic cultivation techniques employed were those of Hungate (4) as modified by Bryant (5) and Balch and Wolfe (6). The medium was similar to that described previously (2) except that the yeast extract component was reduced to 0.02%. Inoculations, HPLC sample collection and culture transfers were performed using sterile glass syringes flushed with O_2-free anaerobic gases.

Transformation of Fluorophenols: Duplicate cultures containing fluorophenols (50 μM - 500 μM) with and without phenol (500 μM) were inoculated (1%) with a phenol-grown culture. Transformation of 4-fluorophenol was also examined using a 10% inoculum at concentrations ranging from 50 μM to 2 mM in the presence and absence of phenol (500 μM). Culture supernatants were analyzed by HPLC weekly.

HPLC Analysis: Culture supernatants were analyzed by HPLC (280 nm) as previously described (2) except that an CH_3COOH (5%):CH_3CN (2 ml/min) solvent was used in ratios ranging from 83:17 to 87:13. Compounds were tentatively identified by retention time and quantified by comparing the area under the peak to a known amount of authentic compound. A Hewlett-Packard 1090M high performance liquid chromatograph equipped as previously described (3) was used to resolve 3-fluorobenzoate and 4-fluorobenzoate. In this case, samples were acidified with H_3PO_4 after filtering and were analyzed at 226 nm using a CH_3CN:10mM phosphate buffer (pH 4.0) gradient (CH_3CN 20% to 37% from 0 to 3 min). UV spectral analyses of transformation products were also performed under these conditions using the diode array detector (200 nm to 600 nm).

GC-MS Analysis: Culture supernatant (50 ml) was extracted twice with an equal volume of diethyl ether. Extracts were combined and dried with N_2 over anhydrous Na_2SO_4. To prepare trimethylsilyl derivatives, samples were dissolved in 25 μl pyridine; mixed with 50 μl Regisil RC-2 [bis(trimethylsilyl-trifluoroacetamide) containing 1% trimethylchlorosilane (Regis Chemical Co., Morton Grove, Ill.) and heated at 55°C for 15 min. Derivatized samples were analyzed with an M525 gas chromatograph-mass spectrophotometer (Kratos Analytical, Ramsey, N.J.) equipped with a fused silica capillary column [30 m x 0.32 mm (internal diameter)] having a 0.25 μm (film thickness) DB5 stationary phase. Derivatives were separated using a temperature program (90°C to 140°C, 8°C/min). The injection temperature was 250°C and the carrier gas was helium (1-2 ml/min) with splitting (1:20). The GC column interfaced directly with the ion source (210°C) and mass spectra were obtained at an ionizing voltage of 70 eV and an accelerating voltage of 4kV.

RESULTS

Transformation of 2-Fluorophenol. In the presence or absence of phenol, 2-fluorophenol was transformed to a compound tentatively identified as 3-fluorobenzoate by its retention time

under the initial HPLC separation conditions described above. Although 3-fluorobenzoate and 4-fluorobenzoate cannot be resolved under these separation conditions, 4-fluorobenzoate, unlike the transformation product, absorbed very weakly at 280 nm. Trimethylsilyl derivatives of culture supernatant ether extracts were analyzed by GC-MS to confirm the identity of the transformation product as a fluorobenzoate. A peak detected at m/e 212, indicated the presence of the trimethylsilyl derivative of a fluorobenzoate (Fig. 1). Under these conditions resolution of 3-fluorobenzoate and 4-fluorobenzoate trimethylsilyl derivatives was not achieved. Using the HPLC gradient separation described above, resolution of 3- and 4-fluorobenzoate was achieved (Fig. 2). As a result, it was confirmed that only 3-fluorobenzoate was present in the ether extract (Fig. 2). Spectral analysis under these separation conditions indicated that the UV spectrum of the transformation product was identical to that of authentic 3-fluorobenzoate (See insert Fig. 2).

Identification of the transformation product as 3-fluorobenzoate allowed us to quantify the product and follow the time course of transformation (Fig. 3). In the presence of phenol (500 μM), 2-fluorophenol was stoichiometrically transformed to 3-fluorobenzoate without a lag period. A rapid initial transformation resulted in 85% of the 2-fluorophenol being converted within three weeks. Thereafter, transformation occurred at a slower rate resulting in complete conversion of

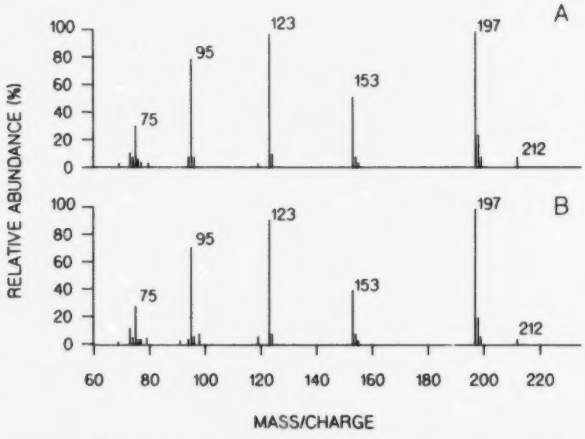

Fig. 1. Mass spectrum of 3-fluorobenzoate (A) compared to that of the transformation product formed from 2-fluorophenol (B).

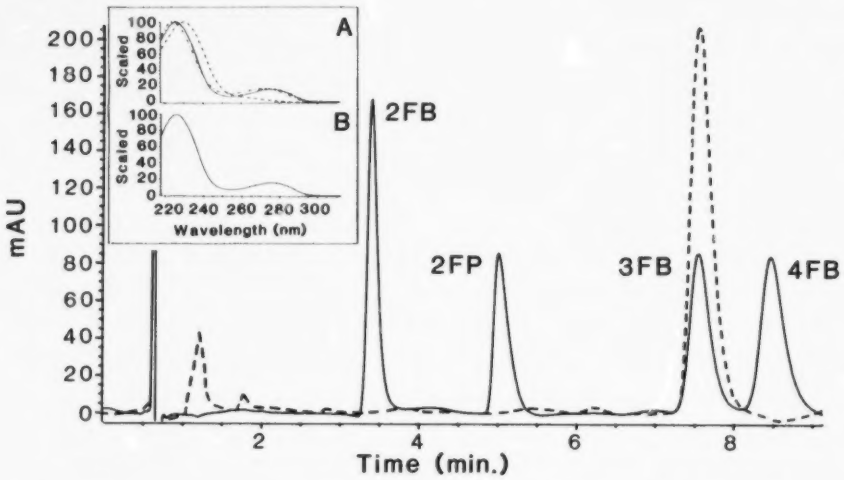

Fig. 2. Chromatography of the transformation product formed from 2-fluorophenol (dashed line) compared to that of the three fluorobenzoate isomers (solid line) using the gradient buffer described in Materials and Methods. Insert: UV spectra (A) of 2-fluorobenzoate (-•-), 3-fluorobenzoate (—) and 4-fluorobenzoate (---) compared to that of (B) the transformation product from 2-fluorophenol.

2-fluorophenol (100 μM) within 5 weeks. 3-Fluorobenzoate was not further metabolized by this consortium.

Transformation of 2-fluorophenol occurred at all concentrations tested (50 μM - 500 μM), but the presence of phenol (500 μM) tended to enhance the rate and degree of transformation (unpublished data). Transformation of phenol to benzoate occurred in the presence of 2-fluorophenol, but

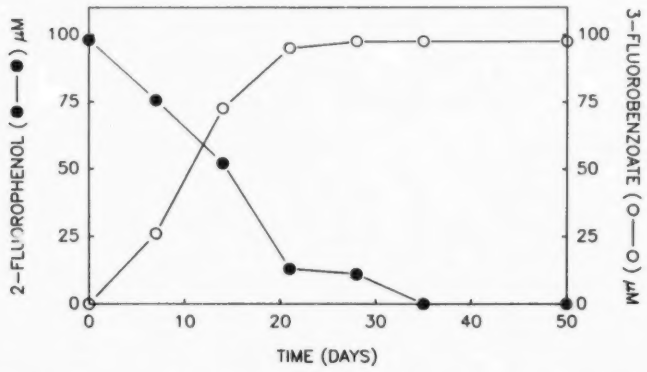

Fig. 3. Time course of the transformation of 2-fluorophenol to 3-fluorobenzoate by an anaerobic phenol-degrading consortium.

concentrations ≥ 250 μM inhibited the rate of phenol transformation (unpublished data).

Transformation of other Fluorophenols. In the presence of phenol, a small amount of 3-fluorophenol (2-3%) was transformed to a single fluorobenzoate product that was identified by retention time and UV spectrum as 2-fluorobenzoate (unpublished data). As a result of limited transformation, we were unable to determine the stoichiometry of this transformation. Despite the low concentration of 2-fluorobenzoate formed, it was easily distinguished from the other isomeric fluorobenzoates (Fig. 2). The 2-fluorobenzoate product was not further metabolized. Transformation was not observed in the absence of phenol. 4-Fluorobenzoate was not detected as a product of 3-fluorophenol transformation in culture supernatant or ether extracts.

In contrast to 2- and 3-fluorophenol, 4-fluorophenol was not transformed at any concentration tested (50 μM - 2 mM) either in the presence or absence of 500 μM phenol (unpublished data).

DISCUSSION

Since phenol and benzoate are symmetrical molecules, asymmetrical fluorinated analogues of phenol allowed us to determine the position at which carboxylation occurred during the transformation of phenol to benzoate under methanogenic conditions. As illustrated in Figure 4, the transformation pattern observed with the three isomers of fluorophenol could only result from carboxylation at the position para to the

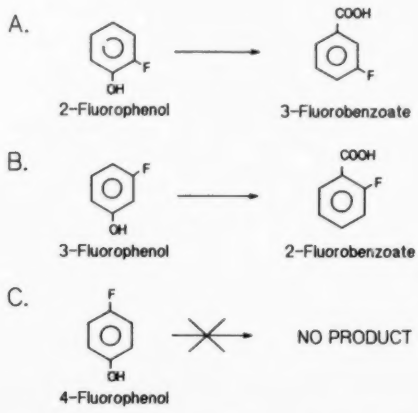

Fig. 4. Products which result from the transformation of monofluorophenols to monofluorobenzoates via para-carboxylation.

phenolic hydroxyl group. The stoichiometric transformation of 2-fluorophenol to 3-fluorobenzoate indicated that carboxylation occurred at either the ortho or para position. Detecting 2-fluorobenzoate as the only product of 3-fluorophenol transformation and observing no transformation of 4-fluorophenol eliminated the possibility of an ortho carboxylation leaving para-carboxylation as the only feasible alternative. This type of carboxylation is analogous to the chemical carboxylation of phenol used industrially (Kolbe-Schmitt reaction) in which salicylate and para-hydroxybenzoate are formed via ortho and para carboxylation, respectively. Recently, an anaerobic bacterium was reported to degrade hydroquinone and gentisic acid forming acetate and benzoate as products (11). The authors suggested that hydroquinone was degraded via gentisic acid as a result of an intermediate ortho carboxylation. Interestingly in the case of hydroquinone, the para position is blocked by a hydroxyl group making carboxylation at the ortho position the only possible alternative.

In preliminary experiments to confirm our finding, we have investigated the transformation of 3-fluorosalicylate and 3-fluoro-4-hydroxybenzoate which are fluorinated analogues of the intermediates which would result from ortho or para carboxylation, respectively. Only in the case of 3-fluoro-4-hydroxybenzoate was 3-fluorobenzoate formed (unpublished data). By analogy, our data suggest that phenol is carboxylated to para-hydroxybenzoate and subsequently dehydroxylated to benzoate during the anaerobic transformation of phenol to benzoate (Fig. 5). Similarly, Tschech & Fuchs (8) concluded from preliminary evidence that para-hydroxybenzoate was the first intermediate in anaerobic phenol degradation by a denitrifying pseudomonad. However, para-hydroxybenzoate was not subsequently dehydroxylated. In contrast, Knoll & Winter (7) have concluded

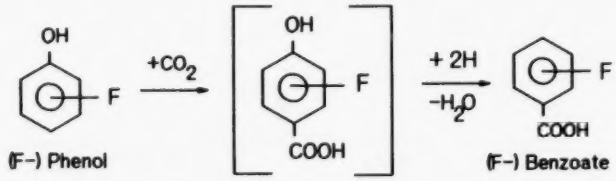

Fig. 5. Possible pathway for the transformation of phenol/fluorophenols to benzoate/fluorobenzoates.

that para-hydroxybenzoate was not an intermediate in the conversion of phenol to benzoate by their methanogenic consortium despite detecting both phenol and benzoate during para-hydroxybenzoate metabolism. Dehydroxylation of para-hydroxybenzoate to benzoate has been reported under methanogenic (9; M.D. Rivera et al., Abstr. Annu. Meet. Am. Soc. Microbiol., 1989, Q5, p. 330), and sulfate-reducing (9) conditions and for a facultative, nitrate-reducing bacterium (10) indicating that this reaction can occur under a variety of anaerobic conditions. Further studies are underway to confirm the role of para-hydroxybenzoate in the metabolism of phenol by our consortium.

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